REMARKS

With this amendment, claim 1 has been amended. Support for the amendments is found in the existing claims and the specification as discussed below. Accordingly, the amendments do not constitute the addition of new matter. Applicant respectfully requests the entry of the amendments and reconsideration of the application in view of the amendments and the following remarks.

Rejection under 35 U.S.C. § 112, second paragraph

Claims 1-5, 9, and 10 are rejected under 35 U.S.C. § 112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The Office Action states that claim 1 is indefinite as it is not clear whether the salt concentration refers to the salt concentration of the buffer or the salt concentration of the fraction obtained from the gel filtration column or both.

Claim 1 has been amended to move the "wherein clause" from line 8 to the "dissolving" step of lines 3-4. The clause has also been amended to: "salt concentration of the buffer" to clarify that the salt concentration recited refers to the buffer. Support for the amendment is found in the present specification at page 5, last 3 lines to page 6, line 2.

In view of Applicants' amendment, reconsideration and withdrawal of the above ground of rejection is respectfully requested.

Rejection under 35 U.S.C. § 103(a)

Claims 1-5, 9, and 10 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Burdick, et al. (EP 0 393 744 A1) in view of Akane, et al. (Biotechniques (1994) 16 (2): 235, 237, 238).

Burdick, et al. teach isolation of nucleic acids from a sample using PCR. However, Burdick, et al. do not teach either the claimed salt concentration or gel filtration. Akane, et al. is cited in to show the use of gel filtration in PCR as a means to remove degraded DNA.

First, claim 1 has been amended as discussed above to clarify that the salt concentration of 0.5 to 2 M refers to the salt concentration of the buffer as discussed above. Neither of the cited references teach the salt concentration of 0.5 to 2 M claimed by Applicants.

It was previously argued that one of ordinary skill in the art would not attempt to use salt in the recited concentration range as it was well known that high salt concentration inhibits the activity of the polymerase and consequently, the PCR reaction. In support, Applicants provided a reference (Chien, et al. (1976) Journal of Bacteriology 127(3):1550; Attachment A, resubmitted herewith), and directed the Examiner's attention to page 1554, left column, lines 13-15 from the bottom which teaches inhibition of polymerase by salts of a monovalent cation such as NaCl and KCl at concentrations above 100 mM.

In the Response to Arguments under item 6 on page 9, the Examiner states that the above argument regarding inhibition of the polymerase is not persuasive :

> because Burdick teaches diluting the purified nucleic acid approximately 10-fold before conducting PCR amplification (see column 14, lines 45-58). Therefore, when practicing the method suggested by the combined teachings of Burdick and Akane, optimization of the NaCl concentration to values within the claimed range (e.g. 0.5 M-1.0 M) would not result in a final salt concentration inhibitory to Taq DNA polymerase activity...

Applicants respond that since the salt concentration of 1.0 to 2.0 M (as well as 0.5-1.0 M) is included in the claimed range of claim 1 of the presently claimed invention., a 10-fold dilution would be in the range to inhibit Taq polymerase taught by Chien, et al. That is, if a 1.0 M salt solution is diluted 10 fold, the result is a 100 mM salt solution which inhibits Taq Polymerase according to Chien, et al.

Regarding the lower end of the range, (0.5- 1.0M) lower dilutions, such as a 5-fold dilution, would inhibit. Accordingly, one of ordinary skill in the art would avoid the claimed range of salt concentrations because of the risk in inhibiting the polymerase as evidenced by Chien, et al.

The second argument put forward by Applicants in the previous response pertained to the advantage of using a salt or salts to disrupt binding between DNAs and binding between DNA and proteins in order to extract the DNA from the cellular material and the DNA-protein complexes without the use of organic solvents such as ethanol and phenol/chloroform extraction

and without repeated centrifugation steps (as discussed in the specification at page 2, paragraph 2 to page 3, paragraph 1). Such advantages could not previously be realized because the presence of the salt is a hindrance when subsequently performing the PCR reaction. Burdick, et al. rely upon both heat and salt to extract the DNA (col. 14, lines 35-40) and the salt concentration taught by Burdick, et al. is much lower than claimed by Applicants. Burdick, et al avoid a higher salt concentration to prevent inhibition of the polymerase and the PCR reaction. Neither Akane, et al. nor Burdick, et al. teach removal of the salt after the DNA extraction and before performing PCR.

Applicants respectfully request reconsideration of this argument in light of the claim amendments. Claim 1 has been amended to make clear that the salt concentration recited refers to the salt in the buffer as discussed above in response to the rejection under 35 U.S.C. § 112, 2nd paragraph.

In response to the Examiner's comment that "the features upon which Applicant relies...are not recited in the rejected claims" (Office Action page 10, first full paragraph), Claim 1 has been further amended to recite "removing PCR inhibitory substances by subjecting the heated solution to gel filtration". Support is found in the present specification at page 6, last paragraph. Accordingly, reconsideration of these arguments in light of the amendments is respectfully requested.

In the Office Action at page 5, last paragraph, the Examiner cites M.P.E.P. 2144.05 as teaching that "Generally, differences in concentration or temperature will not support the patentability of subject matter encompassed by the prior art unless there is evidence indicating such concentration or temperature is critical". Accordingly, Applicants present herewith evidence of criticality for the claimed range of monovalent salt of 0.5 to 2.0 M. Please see the partial translation and original document of Uritani, I, et al., Biochemical Experimental Method 22 in "Chromatin Experimental Method" (Japan Scientific Press), page 175, line 7 to page 179, line 28, November 30, 1988, submitted herewith as Attachment B.

Chromatin is a complex of DNA and protein. DNA is known to exist in nuclei, bound to histone protein.

In order to amplify DNA by PCR, it is necessary to separate the DNA from the histone proteins, so that the histones do not interfere with primer binding. As the salt concentration of

chromatin solution increases, the histone is separated from DNA. In the claimed method of the invention, a salt concentration of 0.5 to 2 M is critical to separate the DNA from the histone protein.

Uritani, et al. (Attachment A) describes that 0.6 M NaCl dissociates histone from DNA (page 2, line 16 of the partial translation). Uritani, et al. (Attachment B) further describe that with increase of NaCl concentration from 0.6 to 2.0 M, the histone dissociates from the DNA, with the result that the peaks (ring-current shifted peaks) appear in the region of 0 to 1 ppm (c to e in FIG. V-10; see page 8, lines 22-24 of the partial translation).

Clearly, the claimed range of salt concentration is critical to the practice of the presently claimed invention in order to provide separation of the protein from DNA to be used for PCR.

In view of Applicants' amendments and arguments, reconsideration and withdrawal of the above ground of rejection is respectfully requested.

Rejection under 35 U.S.C. § 103(a)

Claims 1-5, 9, and 10 are rejected under 35 U.S.C. § 103 (a) as being unpatentable over Miller, et al. (Nucleic Acids Research (1988) 16(3):1215), in view of Sparkman, et al. (Journal of Neurogenetics (1985) 2: 345-363) and further in view of Goldenberger, et al. (PCR Methods and Applications (1995) 4:368-370).

Although Miller, et al. describe a salting out procedure for extraction of DNA, Miller, et al. do not teach any of the steps of the claimed method as recited.

Regarding "dissolving the sample in a buffer comprising at least one surfactant and at least one salt of a monovalent cation, wherein the salt concentration of the buffer is 0.5 to 2 M", while Miller, et al. do teach adding a surfactant and a salt of a monovalent cation to the sample, Miller, et al. do not use a salt concentration in the same range as Applicants (0.5 to 2.0 M).

Regarding, "heating the obtained solution at 80 to 100°C;" and "removing PCR inhibitory substances by subjecting the heated solution to gel filtration", Miller et al. do not teach any aspect of these two steps. Miller, et al. teach removal of protein by precipitation with NaCl, not heat. Miller, et al. teach that the isolated DNA samples are used for RFLP analysis, not PCR. Accordingly, Miller, et al. is not concerned with removing PCR inhibitory substances.

Finally, Miller, et al. do not teach "collecting a solution of a fraction containing nucleic acids" (emphasis added) as the fraction containing nucleic acids is collected as an ethanol precipitated pellet, not a solution.

These deficiencies are not corrected by the secondary references. Sparkman, et al are cited for teaching a gel filtration step. However, Sparkman, et al. also are not directed to PCR, and do not teach or suggest "removing PCR inhibitory substances by subjecting the heated solution to gel filtration" as now claimed. While Sparkman, et al. do teach gel filtration, this reference is silent regarding any PCR considerations. Furthermore, Sparkman, et al. do not teach or suggest the use of a monovalent cation at a concentration of 0.5 to 2.0 M and heat of 80 to 100°C to precipitate DNA. Rather Sparkman, et al. teach the use of organic solvents to isolate plasmid DNA (see page 349, "*Isolation of plasmid DNA*"). As in Miller, et al. above, Sparkman, et al. do not teach isolation of "a solution of a fraction containing nucleic acids" (emphasis added) as Sparkman, et al. teach precipitation with ammonium acetate/ethanol (see page 349, "*Isolation of plasmid DNA*").

Goldenberger, et al. teaches a method of extraction of DNA for PCR using proteinase K and heat. However, Goldenberger, et al. do not teach the use of "salt of a monovalent cation, wherein the salt concentration of the buffer is 0.5 to 2 M" and does not teach the use of gel filtration.

Accordingly, the references taken together as a whole do not teach or suggest all of the elements of the claimed invention as none of the cited references teach the use of a salt of a monovalent cation at a concentration of 0.5 to 2 M which is a critical limitation.

In the Office Action at page 8, the Examiner cites M.P.E.P. 2144.05 as teaching that "Generally, differences in concentration or temperature will not support the patentability of subject matter encompassed by the prior art unless there is evidence indicating such concentration or temperature is critical". Accordingly, Applicants present herewith evidence of criticality for the claimed range of monovalent salt of 0.5 to 2.0 M.. Please see the partial translation and original document of Uritani, I, et al., Biochemical Experimental Method 22 in "Chromatin Experimental Method" (Japan Scientific Press), page 175, line 7 to page 179, line 28, November 30, 1988, submitted herewith as Attachment B.

Chromatin is a complex of DNA and protein. DNA is known to exist in nuclei, bound to histone protein.

In order to amplify DNA by PCR, it is necessary to separate the DNA from the histone proteins, so that the histones do not interfere with primer binding. As the salt concentration of chromatin solution increases, the histone is separated from DNA. In the claimed invention, a salt concentration of 0.5 to 2 M is critical to separate the DNA from the histone protein.

Uritani, et al. (Attachment B) describes that 0.6 M NaCl dissociates histone from DNA (page 2, line 16 of the partial translation). Uritani, et al. (Attachment A) further describe that with increase of NaCl concentration from 0.6 to 2.0 M, the histone dissociates from the DNA, with the result that the peaks (ring-current shifted peaks) appear in the region of 0 to 1 ppm (c to e in FIG. V-10; see page 8, lines 22-24 of the partial translation).

Clearly, the claimed range of salt concentration is critical to the practice of the presently claimed invention in order to separate protein from nucleic acid in the sample.

Also, Applicants again argue that one of ordinary skill in the art would not attempt to use salt in the recited concentration range as it was well known that high salt concentration inhibits the activity of the polymerase and consequently, the PCR reaction. In support, Applicants provide the attached reference which was also provided in the previous response (Chien, et al. (1976) Journal of Bacteriology 127(3):1550; Attachment A). The Examiner's attention is directed to page 1554, left column, lines 13-15 from the bottom) which teaches inhibition of polymerase by salts of a monovalent cation such as NaCl and KCl at concentrations above 100 mM. Accordingly, one of ordinary skill in the art would not have used salt of a monovalent cation in the claimed concentration range at the time of the claimed invention.

As explained previously, the claimed method is based upon the use of a salt or salts to disrupt binding between DNAs and binding between DNA and proteins in order to extract the DNA from the cellular material and the DNA-protein complexes without the use of organic solvents such as ethanol and phenol/chloroform extraction and without repeated centrifugation steps as discussed in the specification at page 2, paragraph 2 to page 3, paragraph 1. Although the method relies upon the salt to extract the DNA, the presence of the salt is a hindrance when subsequently performing the PCR reaction and must be removed as a contaminant. Although Miller, et al. teach the use of relatively high salt to extract the DNA, Miller, et al. do not provide

any means for removal of the salt after the DNA extraction and before performing PCR because Miller, et al pertains to RFLP mapping using restriction enzymes, not PCR. Without the removal of the salt, polymerase and the PCR reaction would be inhibited. However, this problem is addressed by Applicants by the use of a gel filtration step to desalt the sample so that PCR can be performed successfully.

In view of Applicants' amendments and arguments, reconsideration and withdrawal of the above ground of rejection is respectfully requested.

No Disclaimers or Disavowals

Although the present communication may include alterations to the application or claims, or characterizations of claim scope or referenced art, the Applicants are not conceding in this application that previously pending claims are not patentable over the cited references. Rather, any alterations or characterizations are being made to facilitate expeditious prosecution of this application. The Applicants reserve the right to pursue at a later date any previously pending or other broader or narrower claims that capture any subject matter supported by the present disclosure, including subject matter found to be specifically disclaimed herein or by any prior prosecution. Accordingly, reviewers of this or any parent, child or related prosecution history shall not reasonably infer that the Applicants have made any disclaimers or disavowals of any subject matter supported by the present application.

CONCLUSION

In view of Applicants' amendments to the claims and the foregoing Remarks, it is respectfully submitted that the present application is in condition for allowance. Should the Examiner have any remaining concerns which might prevent the prompt allowance of the application, the Examiner is respectfully invited to contact the undersigned at the telephone number appearing below.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: Sept. 15, 2008

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ATTACHMENT A

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Deoxyribonucleic Acid Polymerase from the Extreme Thermophile *Thermus aquaticus*

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A stable deoxyribonucleic acid (DNA) polymerase (EC 2.7.7.7) with a temperature optimum of 80°C has been purified from the extreme thermophile *Thermus aquaticus*. The enzyme is free from phosphomonoesterase, phosphodiesterase, and single-stranded exonuclease activities. Maximal activity of the enzyme requires all four deoxyribonucleotides and activated calf thymus DNA. An absolute requirement for divalent cation cofactor was satisfied by Mg^{2+} or to a lesser extent by Mn^{2+}. Monovalent cations at concentrations as high as 0.1 M did not show a significant inhibitory effect. The pH optimum was 8.0 in tris(hydroxymethyl)aminomethane-hydrochloride buffer. The molecular weight of the enzyme was estimated by sucrose gradient centrifugation and gel filtrations on Sephadex G-100 to be approximately 63,000 to 68,000. The elevated temperature requirement, small size, and lack of nuclease activity distinguish this polymerase from the DNA polymerases of *Escherichia coli*.

Studies on the biosynthesis of deoxyribonucleic acid (DNA) in *Escherichia coli* during the last 20 years have contributed substantially to our knowledge of this fundamental cellular process in bacteria. The original enzyme DNA polymerase I, which was thought to be involved in DNA synthesis, has been purified and charcterized in a series of studies reported by Arthur Kornberg and his colleagues (2, 5, 8–10, 16). These studies included such aspects as the physical and chemical properties of the purified enzyme, the primer-template DNA required for the reaction, the product DNA, and the reaction mechanisms of the polymerization of deoxyribonucleotides into DNA.

In 1969 DeLucia and Cairns (7) discovered an amber mutant, PolA, of *E. coli* W3110 that appeared to lack DNA polymerase activity but synthesized DNA normally. The isolation of this mutant not only casts serious doubt on the role of DNA polymerase I in in vivo DNA replication, but also pointed out the complexity of this cellular process. As a result, the discovery of this mutant led to the search for other replication enzymes, and two new polymerases have since been isolated and characterized (14, 15, 19, 20, 31).

In contrast to the extensively researched replication phenomena in mesophiles, few attempts have been made to isolate DNA polymerases from thermophiles (27). Thermophiles are ubiquitous in nature, and many prokaryotic species thrive at temperatures above 45°C. Attempts to explain the proliferation of life at temperatures that destroy most mesophilic cellular components have resulted in widespread investigation in the last 25 years, with many of these studies focusing on protein structure. In this communication the purification and characterization of a thermophilic polymerase will be discussed in relation to what is known about DNA polymerases from mesophilic microorganisms.

MATERIALS AND METHODS

Strain. *Thermus aquaticus* YT-1 used in these experiments was supplied by Paul Ray, Burroughs Wellcome.

Culture medium. Cells were grown in a defined mineral salts medium containing 0.3% glutamic acid (which served as both a carbon and nitrogen source), which was supplemented with biotin and thiamin (0.1 mg/liter each) and nicotinic acid (0.05 mg/liter). The salts included in 1 liter of medium were: nitrilotriacetic acid, 100 mg; $CaSO_4 \cdot 2H_2O$, 60 mg; $MgSO_4 \cdot 7H_2O$, 100 mg; NaCl, 8 mg; KNO_3, 103 mg; $NaNO_3$, 689 mg; $ZnSO_4$, 5 mg; H_3BO_3, 5 mg; $CuSO_4$, 0.16 mg; $NaMoO_4 \cdot 2H_2O$, 0.25 mg; $CoCl_2$, 0.4 mg; $FeCl_3$, 0.28 mg; $MnSO_4 \cdot H_2O$, 22 mg; and Na_2HPO_4, 110 mg. The pH of the medium was adjusted to 8.0 with NaOH.

Growth conditions. Cells were grown initially in 500-ml Erlenmeyer flasks at 75°C in a New Brunswick water bath shaker. When the cultures reached a density of approximately 170 Klett units, 1 liter of these cells was transferred to 16-liter carboys, which were placed in hot-air incubators. In place of shaking, sterile air was bubbled through the cultures, and the temperature was maintained at 75°C. The cells were allowed to grow for 20 h before they were collected with a Sharples continuous-flow centrifuge.

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Preparation of enzyme extract. Subsequent to collection, the cells were suspended in standard KP buffer (0.02 M potassium phosphate [pH 7.5], 8% [vol/vol] glycerol, 0.005 M ethylenediaminetetraacetate [EDTA], 0.01 M NaN_3, 0.01 M KCl). The cells were ruptured in a Branson 20-kc Magnetostrictive ultrasonic oscillator, operated at 3.5 A for 45 s. The extracts were then spun in a Sorvall RC-2B centrifuge at 17,300 × *g* for 20 min. The supernant fluid served as the crude extract.

Protein determinations. Protein concentrations were determined by the method of Lowry et al. (23).

Enzyme assays. (i) Duplicate DNA polymerase assays were carried out in disposable glass test tubes. The reaction mixture (125 μl) contained: tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 8.0), 25 mM; 2-mercaptoethanol, 1 mM; $MgCl_2$, 10 mM; KCl, 25 mM; deoxyadenosine 5'-triphosphate (dATP), deoxycytidine 5'-triphosphate (dCTP), deoxyguanosine 5'-triphosphate (dGTP), and ^{3}H-labeled thymidine 5'-triphosphate ([^{3}H]dTTP; specific activity, 38.8 mCi/mmol), each 0.15 mM; and activated calf thymus DNA (prepared by the method of Loeb et al. [22]), 12.5 μg. After 30 min of incubation at 80°C in sealed tubes, the assay was stopped by chilling the tubes in an ice bath for a few minutes. Samples of 100 μl were then pipetted onto 25-mm Schleicher and Schuell filter paper disks and immediately dropped into ice-cold 10% trichloroacetic acid containing 0.1 M sodium pyrophosphate for at least 1 h. This was followed by two changes of 5% trichloroacetic acid. The first change contained 0.1 M sodium pyrophosphate. Finally, the disks were run through 30-min washes in ether-ethanol (1:1, vol/vol), ether-ethanol (3:1, vol/vol), and ether. The disks were air dried, and the amount of [^{3}H]dTTP incorporated into an acid-insoluble product was measured in a Packard scintillation spectrometer with a 5% counting efficiency for ^{3}H under these experimental conditions. One unit of enzyme is defined as the amount of enzyme that will incorporate 10 nmol of [^{3}H]dTTP into acid-insoluble material at 80°C in 30 min.

(ii) The exonuclease assays were conducted in a reaction mixture containing 25 mM Tris-hydrochloride (pH 8.0); 1 mM 2-mercaptoethanol; 10 mM $MgCl_2$; 12 μg of ^{3}H-labeled DNA prepared by the method of Reuben et al. (25), and approximately 0.02 U of polymerase in a final volume of 125 μl. After 30 min of incubation at 80°C, the tubes were chilled and the acid-insoluble material was measured by the filter paper disk method as described previously for the polymerase. The amount of acid-soluble counts released was used as a measure of exonuclease activity.

(iii) Alkaline phosphomonoesterase activity was assayed in a reaction mixture containing 1 mM *p*-nitrophenyl phosphate, 0.1 M Tris-hydrochloride buffer (pH 8.0), and approximately 0.02 U of polymerase in a final volume of 0.5 ml. After 20 min at 80°C, the reactions were stopped by chilling the tubes and then adding 0.5 ml of 0.1 N NaOH. The formation of *p*-nitrophenol was determined spectrophotometrically at 410 nm. *E. coli* alkaline phosphatase was used as a control. In addition, the phosphomonoesterase activity was assayed in the standard DNA polymerase assay mixture.

(iv) Alkaline phosphodiesterase I was assayed in a manner similar to that for alkaline phosphatase, except that 1 mM thymidine 5-monophospho-*p*-nitrophenyl ester was used as a substrate. Phosphodiesterase II activity was measured with 1 mM thymidine 3-monophospho-*p*-nitrophenyl ester as substrate and 0.1 M sodium succinate buffer, pH 6.5. In addition, both substrates were assayed in the standard DNA polymerase assay mixture.

Polyacrylamide gel electrophoresis. Disc-gels were prepared by the method of Davis (6). The separating gel (8.0 by 0.5 cm) contained 7% acrylamide, whereas the spacer gel (1.5 by 0.5 cm) contained 2% acrylamide. Electrophoresis in a Canalco model 66 electrophoresis bath was carried out at room temperature in 2.5 mM Tris-1.9 mM glycine buffer (pH 9.5) for 2.5 h at 2 mA. The gels were stained with 0.1% Coomassie brillant blue in 7% acetic acid and subsequently destained by diffusion in 7% acetic acid. For the recovery of the DNA polymerase activity from the gel, the gels were prepared in exactly the same manner, except that there was a 1-h electrical prerun to remove ammonium persulfate, which inhibits the enzymatic activity of the polymerase. Samples were then applied and electrophoresis was continued. The gels were removed from the tubes, sliced into 2-mm disks, and placed directly into tubes containing the polymerase assay mixture. No additional process for the elution of the enzyme off gel was required since the incubation temperature of the polymerase was 80°C.

Sucrose gradient centrifugation. Purified DNA polymerase (fraction IV, 50 μl) and bovine serum albumin (BSA) (2 mg) were layered on a linear sucrose gradient of 6 to 20%. The gradient also included 0.01 M Tris (pH 7.5), 0.001 M EDTA, 8% glycerol, 0.01 M NaN_3, and 0.5 M NaCl. Centrifugations were preformed in a Spinco SW50L rotor in a preparative Beckman ultracentrifuge at 38,000 rpm for 14 h at 4°C. Fractions (0.2 ml) were collected from the top of the gradient with an ISCO density gradient fractionator. BSA was assayed for absorbance at 280 nm, and the DNA polymerase was assayed by enzymatic activity. Approximate molecular weight for the DNA polymerase was determined by the method of Martin and Ames (24).

Molecular weight estimation by gel filtration. A column (Pharmacia K 16/70) was packed with Sephadex G-100 that had been preequilibrated in standard KP buffer containing 0.5 M NaCl and 0.01 M NaN_3. The void volume was determined by using blue dextran, and three proteins of known molecular weight were used as standards: BSA, 68,000; ovalbumin, 43,000; and lysozyme, 14,300. The approximate molecular weight of the DNA polymerase was determined in reference to a linear plot of the three standard proteins by the method of Andrews (1).

Chromatographic procedures. All chromatography was conducted at room temperature. Pharmacia chromatographic columns (K9, 15, 16, 50) and accessories were used. Flow rates of 40 ml/h for diethylaminoethyl (DEAE)-Sephadex and phosphocellulose

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and 6 ml/h for DNA cellulose were maintained through the use of a Harvard multispeed peristaltic pump. Fractions were collected with a Büchner automatic fraction collector. Phosphocellulose (Whatman grade P-1) was prepared by the method of Loeb (21), and the DNA cellulose was prepared by the method of Litman (18).

Chemicals and reagents. Calf thymus DNA type V, the four deoxyribonucleotides, pancreatic deoxyribonuclease (DNase), and BSA were purchased from Sigma Chemical Co. Ribonuclease (RNase) A and T, were purchased from Worthington Biochemicals Corp. The lysozyme and ovalbumin came from Schwarz/Mann. The radioactive isotopes [^{3}H]dTTP and [^{3}H]thymine were obtained from Schwarz Bio-Research, and [^{3}H]dATP was from New England Nuclear Corp. Chemicals and reagents for general use were of analytical grade and were purchased from Fisher Scientific Co.

RESULTS

Purification of DNA polymerase from *T. aquaticus*. For the purification, crude extract was prepared from 16 liters of cells as described above. Fraction I, 176 ml of sonically treated crude extract, was placed on a DEAE-Sephadex A-50 column that had been equilibrated previously with standard KP buffer. The column used was a Pharmacia K50/60 with flow adaptors and a set bed volume of 800 ml. A linear 2-liter gradient of 0.01 to 0.4 M KCl was used in standard KP buffer. The DNA polymerase eluted at a salt concentration of approximately 0.15 M. The pooled fractions were dialyzed against standard KP buffer (fraction II) and placed on a phosphocellulose column with a set bed volume of 40 ml packed in a K15/30 Pharmacia column. The peak of activity eluted at a salt concentration of 0.15 M KCl in a 400-ml linear gradient (0.01 to 0.4 M KCl in standard KP buffer). The pooled fractions were then dialyzed against 1 mM Tris-hydrochloride (pH 8.0) containing 1 mM β-mercaptoethanol, 0.1 M NaCl, 8% (vol/vol) glycerol, and 1 mM EDTA (fraction III). After dialysis, 500 g of BSA per ml was added to the enzyme preparation. The inclusion of BSA in this step of purification is necessary since its omission results in complete loss of polymerase activity. The enzyme preparation was then loaded on a preequilibrated DNA cellulose column and washed with 4 bed volumes of Tris buffer containing 1 mM EDTA, 8% (vol/vol) glycerol, 1 mM β-mercaptoethanol, and 500 μg of BSA per ml. The major peak of polymerase activity was eluted with 0.6 M NaCl. The peak fractions were pooled and dialyzed against KP buffer (fraction IV). A summary of the purification is shown in Table 1. The specific activity of fraction IV could not be determined due to the presence of BSA. Attempts were made to remove the BSA from the enzyme preparation on phosphocellulose and were partially successful. The major problem was the loss of over 95% of the activity during the process; therefore, we used fraction IV for the characterization of the enzyme since it was stable at −4°C for at least 3 months.

Polyacrylamide gel electrophoresis. Since BSA had been added to the enzyme preparation, we had no means of estimating the purity of the final enzyme preparation based on specific activity. Therefore, to get an estimate of purity, polyacrylamide gel electrophoresis was carried out. The enzyme was first passed through hydroxylapatite, to remove contaminating DNA eluted from the DNA cellulose, before it was loaded onto the gels. The results can be seen in Fig. 1, where BSA was run as a control (gel A). The enzyme preparation (gel B) was run in duplicate, since one gel was stained with Coomassie brilliant blue and the other was sliced and assayed for polymerase activity (plotted below gel B). The peak of activity corresponds to one of four major bands. Two of the four major bands appear to represent BSA, but it also should be mentioned that protein contaminants in the final preparation could migrate along with them and thus be hidden from detection.

Contaminating enzyme activities. Fraction IV was tested for a number of possible contaminating activities that might interfere with the DNA polymerase reaction. DNA polymerase (0.02 U per reaction tube) was assayed as described above for alkaline phosphomonoesterase and acid phosphodiesterases I and II. All of these activities were at background levels.

Exonuclease assays were conducted as described previously, using single-stranded (heat-denatured) ^{3}H-labeled λ DNA as a substrate. Experimental conditions and the amount of radioactive counts solubilized after 30 min of incubation are listed in Table 2. Only the pancreatic DNase I control (assayed at 37°C) was shown to be effective in solubilizing the ^{3}H-labeled λ DNA. Under these experimental conditions, the final enzyme preparation appears to be free from exonuclease activity in the presence and absence of ATP.

Temperature optimum. The effect of temperature on the catalytic activity of the enzyme can be seen in Fig. 2. The temperature optimum for the incorporation of [^{3}H]dTTP into acid-insoluble material was 80°C. At this temperature, the amount of incorporation was 10 to 15 times greater than that which was measured at 37°C. This optimum profile may reflect melt-

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TABLE 1. *Summary of the purification procedure*[a]

Fraction	Vol (ml)	Total polymerase activity (U)	Total protein (mg)	Yield of activity (%)	Sp act (U/mg)	Purification (fold)
Crude	176	2,080	975.0		2.13	
DEAE-Sephadex	210	4,613	197.5	221	23.3	10.9
Phosphocellulose	132	1,900	16.0	91	118.7	55.7
DNA-cellulose	63	685		33		

[a] One unit of polymerase equals the incorporation of 10 nmol of [^{3}H]dTTP into acid-soluble material at 80°C in 30 min.

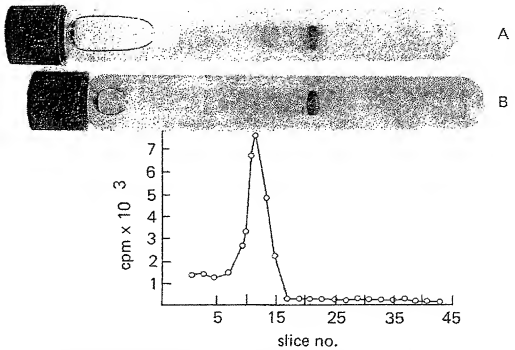

FIG. 1. *Gel electrophoresis of purified enzyme. Gel A, Electrophoresis of 25 μg of BSA at room temperature in 2.5 mM Tris-1.9 mM glycine buffer (pH 9.5). Gel B, Electrophoresis of 50 μl of fraction IV, which was stained for protein. The graph below gel B represents a gel of a size identical to that of B, which was subjected to electrophoresis at the same time but was sliced and assayed for activity as described in the text.*

ing of the template since the melting temperature of the DNA under these experimental conditions is 76°C. Thus, the decrease in catalytic activity above 80°C may be due to the denaturation of the template rather than the DNA polymerase since the enzyme has reduced activity with single-stranded DNA.

pH optimum. The effect of pH on the activity of the enzyme with three different buffer systems can be seen in Fig. 3. The pH optimum for the enzyme is in the range of 7.0 to 8.0 but varies with the buffer used. The highest activity was found with Tris-hydrochloride buffer, pH 7.8. The lowest activity occurred in potassium phosphate buffer, a situation which has been reported for mesophilic polymerases (16, 29).

Effect of divalent cations. This thermophilic DNA polymerase, like all other known polymerases, has an absolute requirement for divalent cations. Optimal activity is obtained with 10 mM Mg^{2+} (Fig. 4). Manganese was only partially effective and showed an optimum of 2 mM. Calcium ion was completely ineffective (data not shown).

Effect of monovalent cations. The addition of relatively low levels of KCl and NaCl stimulates the catalytic activity of the polymerase.

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TABLE 2. *Absence of single-stranded exonuclease specific activity*

Experimental conditions	% of cpm solubilized
Control (−DNA polymerase)[a]	0
DNA polymerase	0
DNA polymerase + 2 mM ATP	0
RNase A[b]	0
Pancreatic DNase I[b]	67.3

[a] The reaction mixture contained 25 mM Tris-hydrochloride (pH 8.0), 1 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 12 μg of single-stranded ^{3}H-labeled λ DNA, and approximately 0.02 U of polymerase in a final volume of 125 μl. The reactions, except where otherwise stated, were run for 30 min at 80°C. The percentage of acid-insoluble counts released was used as a measure of exonuclease activity.

[b] Assayed at 37°C.

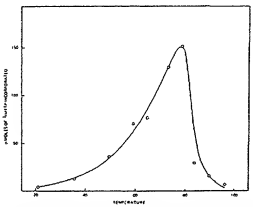

FIG. 2. *Effect of temperature on the activity of the DNA polymerase. DNA polymerase (0.02 U per assay) was incubated for 30 min in the standard reaction mixture at different temperatures. Fractions were then taken, and the conversion of [^{3}H]dTTP into acid-insoluble material was measured.*

The optimal concentrations of NaCl is 40 mM, whereas that of KCl is 60 mM. Above 100 mM, both salts inhibit the activity of the polymerase. These data are in contrast to polymerase I of *E. coli*, which is relatively insensitive to a high salt concentration but shows effects similar to those seen with polymerase II (16).

Requirements for DNA polymerase reaction. Various components of the reaction mixture were removed to see what effect this would have on in vitro DNA synthesis. Low activity in the absence of template DNA, Mg^{2+}, or deoxyribonucleoside triphosphates indicates the need for these components (Table 3).

Molecular weight estimation. (i) Sucrose gradient centrifugation. The approximate molecular weight of the purified DNA polymerase was determined by the method of Martin and Ames (24). The DNA polymerase activity peak corresponds to the optical density of the marker BSA (Fig. 5); this would indicate that its approximate molecular weight is about 68,000.

(ii) Gel filtration on Sephadex G-100. A Sephadex G-100 column was standardized and used to determine the molecular weight of the DNA polymerase as described in Materials and Methods. It can be seen in Fig. 6, plotting log molecular weight versus V_e/V_o, that the approximate molecular weight is 63,000.

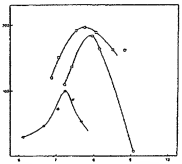

FIG. 3. *Effect of pH on the activity of the DNA polymerase from T. aquaticus. DNA polymerase (0.02 U per assay) was assayed in the standard reaction mixture with various buffers. The reactions were run at 80°C for 30 min, and corrections were made for the temperature coefficients of the various buffers used. Symbols: □, activity in 25 mM Tris-hydrochloride buffer; ○, activity in 25 mM glycine-sodium hydroxide buffer, ●, ●, activity in 25 mM potassium phosphate buffer.*

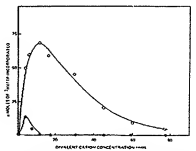

FIG. 4. *Effect of divalent cations on the DNA polymerase. DNA polymerase (0.02 U per assay) in the standard reaction mixture was used, except that the divalent cation concentration was varied as indicated. Symbols: ○, activity in $MgCl_2$, ●, activity in $MnCl_2$.*

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TABLE 3. *Requirements for DNA polymerase reaction*

Reaction components	[^{3}H]dTTP activity incorporated (pmol)	% Control
Complete reaction mixture[a]	102.9	100
$-Mg^{2+}$	6.0	6
−DNA	2.9	3
−dATP, dGTP, dCTP	18.6	18
−dATP	28.2	28
−dCTP	37.5	37
−dGTP	38.9	39
−dTTP[b]	20.7	21

[a] The complete reaction mixture contained in 125 μl: 25 mM Tris-hydrochloride (pH 8.0); 25 mM KCl; 10 mM $MgCl_2$; 1 mM 2-mercaptoethanol, 0.15 mM dATP, dCTP, dGTP, and [^{3}H]dTTP; 12.5 μg of calf thymus DNA; and 0.01 U of enzyme.

[b] [^{3}H]dATP was used instead of [^{3}H]dTTP.

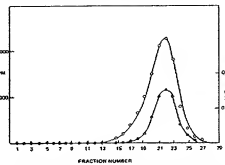

FIG. 5. *Molecular weight determination by sucrose gradient centrifugation. Fraction IV DNA polymerase (50 μl) and BSA (2.0 mg) were layered on a 5-ml linear sucrose gradient of 6 to 20% and run as described in the text. Fractions of 200 μl were collected and assayed for BSA by adsorbance at 280 nm (●) and DNA polymerase by catalytic activity (○).*

DISCUSSION

A stable thermophilic DNA polymerase has been isolated and purified. The gel data indicate that the final sample, although not homogenous, represents a relatively pure fraction containing BSA. Attempts to remove the BSA from the enzyme sample were only partially successful and resulted in extensive loss of the catalytic activity of the DNA polymerase, a situation which could have resulted from the low protein concentration of DNA polymerase after its separation from the BSA. The molecular weight of the enzyme has been estimated by sucrose gradient centrifugation to be 68,000, and by gel filtration it was estimated to be approximately 63,000, two sets of data which are relatively close in agreement. Its size, in relation to the three DNA polymerases from *E. coli*, is relatively small, although a DNA polymerase from *Bacillus subtilis* has been isolated which has a molecular weight of 46,000 (11). It should be pointed out that polymerase I from *E. coli* can be cleaved into two fragments by trypsin, one of molecular weight of 76,000 and the other of 34,000 (4). The large fragment retains the polymerase and 3′→5′ exonuclease activities, whereas the small fragment appears to be responsible for the 5′→3′ exonuclease activity. Thus, the question of whether the DNA polymerase isolated from *T. aquaticus* represents the native form of the enzyme in vivo or is a result of proteolytic cleavage during isolation cannot be answered at this time. The enzyme also appears different from the *E. coli* polymerases in that the final sample (fraction IV) is free from single-stranded exonuclease activity. The enzyme, like mesophilic DNA polymerases, requires all four deoxyribonucleoside triphosphates as well as DNA and Mg^{2+} for good catalytic activity. Omission of any one unlabeled deoxyribonucleoside triphosphate still gives approximately 35% maximal incorporation. This amount is higher than those reported for most bacterial polymerases but is similar to certain mammalian DNA polymerases (26). The incorporation probably represents the addition of two or three dTTP's to complementary ends.

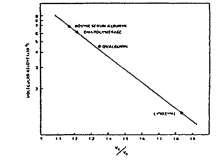

FIG. 6. *Estimation of the molecular weight of the DNA polymerase by gel filtration. A Sephadex G-100 column was run and assayed as described in the text. The molecular weight of the DNA polymerase was determined in reference to the three standard proteins by the method of Andrews (1).*

The most unique feature of this enzyme is its temperature optimum of 80°C, which is 15°C higher than the optimal temperature reported for the DNA polymerase from *Bacillus stearothermophilus* (27). In addition to the scientific interest, this wide temperature range also provides the possibility of using this enzyme in

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gene synthesis in the form of a reverse transcriptase. Other DNA polymerases, such as polymerase I from *E. coli*, were shown under various experimental conditions to synthesize DNA by using ribonucleic acid (RNA) template (13). The DNA products of these DNA polymerases and the reverse transcriptases from tumor viruses appeared to have fallen short of completion of the entire gene (3, 28–30). One possible explanation for this could be interference by the secondary and tertiary structure of the DNA template in the completion of the DNA product. With an enzyme such as a thermophilic DNA polymerase, which has a wide temperature range, one might be able to overcome some of these difficulties, since it is possible to melt out the secondary and tertiary structure of the RNA template at elevated temperatures. We are currently investigating the possibility of using certain purified RNAs as template for this enzyme.

ACKNOWLEDGMENTS

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ATTACHMENT B

A partial translation of Chromatin Experimental Method (Japan Scientific Society Press) (Uritani I. et al, Biochemical Experimental Method 22, page 175, line 7 to page 179, line 28, November 30, 1988)

(ii) Interaction between DNA and protein

There are employed a physicochemical method and a chemical method to analyze an interaction between DNA and histone in a nucleosome. The former includes a nuclear magnetic resonance method (NMR), a thermal melting method, and the like. The latter includes a chemical modification method, a cross-linking method, a method using DNase, and the like. In addition, those methods are also used for analyzing an interaction between a nucleosome and a nonhistone protein, HMG. Regarding the structure of the nucleosome and the interaction between the DNA and histone, please also refer to other documents (Ohba, 1978; McGhee & Felsenfeld, 1980 a; Kornberg & Klug, 1981; Ichimura et al., 1983; Zama et al., 1984).

(a) Bond between DNA and histone

The nucleosome is the basic unit structure of chromatin, and has a structure in which about 200 base pairs of DNAs sinistrorsely wind themselves in two rounds around the outside of a histone octamer formed of respective two molecules of H2A, H2B, H3, and H4, with one molecule of H1 histone bonding with each of the site at a volute tongue and the site at a volute tail. The part in which 146 base pairs of DNAs wind themselves in 1.75 times around the histone octamer

is called a nucleosome core. The DNA linking core particles is called a linker.

The nucleosome core DNA having 146 base pairs has 292 phosphate groups that are negatively charged. On the other hand, in the case of calf thymus, for example, the histone octamer has 220 basic amino acid residues (116 pieces of lysine and 104 pieces of arginine), which are positively charged, and 74 acidic amino acid residues that are negatively charged. That is, the calf thymus has net positive charge corresponding to 146 (220 - 74 = 146). In other words, the histone octamer has the positive charge that is sufficient for neutralizing exactly half of the negative charge possessed by the nucleosome core DNA. Accordingly, the above fact first leads to a presumption that electrostatic interaction is responsible for the bond between histone and the DNA. As the facts supporting the above-mentioned presumption, the following phenomena are, for example, given: (1) 0.6 M NaCl dissociates H1 from DNA, 0.6 M or more NaCl dissociates H2A and H2B from DNA, and 1 M or more NaCl dissociates H3 and H4 from DNA (Ohlenbush et al., 1967; Burton et al., 1978; Ruiz-Carrillo & Jorcano, 1979); (2) DNA and histone are dissociated from each other at a pH of 2 or less; and (3) the bond between DNA and histone is stable in an organic solvent such as urea and ethanol (Olins et al., 1977; Zama et al., 1978 b). However, another interaction also cannot be disregarded. For example, in the presence of urea, histone is dissociated from the DNA at an

extremely low salt concentration (0.2 or less NaCl) (Woodcock & Frado, 1977). This shows that the urea contributes to loosen the structure of the histone octamer, with the result that the bond between the DNA and the histone becomes weak. A hydrophobic interaction and a hydrogen bond dependent on a specific configuration may probably contribute.

In a histone molecule, there is a distribution bias in the molecule of various amino acids. In the N-terminal and the C-terminal, particularly in one third of the N-terminal side of the molecule, the content rate of basic amino acids is high. In the part from the molecular center to the C-terminal side, hydrophobic amino acids and acidic amino acids are abundant. Therefore, the histone molecule has an amino acid composition analogous to an ordinary spherical protein. The histone molecule has a random structure at both terminals reflecting its primary structure and forms a higher-order structure from the molecular center to the C-terminal. The higher-order structure parts of each histone interact with each other to form the histone octamer. Thus, the histone octamer can be roughly divided into a spherical structure part having abundant α helixes and a highly basic terminal chain (long N-terminal chain and short C-terminal chain) part having abundant basic amino acids and having the random structure. Intuitively, the terminal chain is probably a main bonding site of the bond between the DNA and histone. However, the following experiments demonstrate that the

interaction between the spherical structure part of the histone octamer and the DNA is rather rigid. (1) Even if an isolated nucleosome core is subjected to trypsin treatment to remove the histone terminal chain selectively, the histone stably bonds with DNA, and the "particle" structure is maintained (Grigoryev & Krasheninnikov, 1982). (2) Histone which is subjected to trypsin treatment to remove its terminal chain and untreated histone either are eluted from DNA at the same salt concentration as that for naturally occurring chromatin (Palter & Albert, 1979). (3) An NMR method has shown that the bond between the histone terminal chain and DNA is weak (Cary et al., 1978; see Experimental Example 8). In addition, a basic amino acid treated with a chemical modification method has demonstrated that arginine residues in the spherical structure part of an histone octamer tightly bond with DNA (Ichimura et al., 1982; see Experimental Example 9).

A lysine residue in the basic region in the N-terminal side of a core histone is acetylated depending on physiological conditions of cells. The acetylating reaction leads to disappearance of positive charge of an ε-amino group in a side chain of the lysine residue, with the result that a little larger group is introduced. On the other hand, a butyric acid is a histone deacetylase inhibitor, and when nucleosome cores are isolated from cells cultured in a medium containing 5 to 10 mM butyric acids, hyperacetylated core particles can be obtained, the core particles having hyperacetylated

side chains of the lysine residue in the vicinity of the histone N-terminal in the core particles. Investigation of the structure and stability of the hyperacetylated core particles provides information about the interaction between the DNA and histone.

Nuclear magnetic resonance method

When a group of nuclei having a magnetic moment is placed in a static magnetic field, the nuclei are distributed to discrete energy levels by a nuclear Zeeman effect in accordance with magnitude of the magnetic moment and intensity of a magnetic field. When electromagnetic radiation corresponding to the distance between the energy levels is applied, resonance absorption is observed, which is a phenomenon called nuclear magnetic resonance (NMR). For the measurement of the resonance absorption, a sweep method is generally used, in which one of frequency of the electromagnetic radiation applied and magnitude of the static magnetic field is fixed, and the other that is not fixed is continuously changed to record resonance points. However, a pulse Fourier transform method has recently been a main stream, in which a strong pulse magnetic field having a fixed frequency is given to satisfy a resonance condition simultaneously with respect to signals in a wide range, and the time response undergoes Fourier transform using a mini-computer to determine a normal frequency spectrum. Four parameters of an NMR spectrum, a position of an NMR absorption line (chemical shift), an area (intensity), a width, and splitting

provides information about the molecules to be investigated. The position reflects a state of presence of nuclei. The area is proportional to the number of nuclei in the same chemical environment. The width is widened as the degree of freedom of nuclear movement decreases. The splitting occurs by an interaction with a nuclear magnetic moment nearby. Regarding a general method of NMR and an experimental method of a protein and a nucleic acid, please refer to the documents (Arata, 1976; Sora, Kyogoku, 1977; Campbell & Dobson, 1979).

Generally, the part having a random structure in a protein has higher mobility, with the result that resonant nuclei in the part give acute resonant signals. However, when the part gets contact to another molecule and is directly associated with structural formation, the mobility of the part is lowered, with the result that the resonant signals are widened. As described above, there is a bias in an amino acid sequence of a histone molecule. In the basic part in the both molecular terminals, there are many residues such as glycine, serine, and proline, in which α-helixes are rarely formed, in addition to the basic amino acids. On the other hand, in the nonpolar part in the molecular center, there are many aromatic acidic amino acid residues having a nonpolar property, which tend to form the α-helixes. Therefore, observation of the NMR can clarify the issue of what part of the polypeptide chain in histone is associated with the interaction between the histone and the histone

and the interaction between the histone and the DNA.

[Experimental Example 8] Analysis of interaction between DNA and histone by NMR method (Cary et al., 1978)

Respective protons of amino acids in the basic part and the nonpolar part of hitone generate resonance lines on different positions. Using this characteristic, a dissociation process of a histone molecule from DNA with a salt concentration change was tracked by NMR, whereby the bond between DNA and histone was analyzed. A nucleosome core (3 to 5 mg/ml) prepared from calf thymus is dialyzed with a 10 mM phosphate buffer solution (pH of 6.5 to 7.0). A sample solution for NMR measurement is dialyzed for 48 hours with 0.5 ml of D_2O-1 mM phosphate buffer solution (pH of 6.5)-NaCl. The same dialysis is repeated four times, and the sample solution is finally dialyzed with the same D_2O buffer solution in 40-fold volume. The NMR measurement is performed using an apparatus of 270 MHz Fourier transform type. FIG. V-10 shows ^1H-NMR spectrum of the nucleosome core measured under various salt concentrations (0 to 2 M NaCl). The abscissa axis shows a chemical shift, in which the difference between resonance frequency of the proton peak as an object and that of the peak of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as a standard substance is divided by the resonance frequency of the spectrometer, and the obtained value is multiplied by 10^6 to show the value by a ppm unit. The total area of the peak region

of the NMR spectrum (a) of the histone in a core particle decreases by 17 to 20% compared with that of the NMR spectrum (f) of the histone that has been in the state of a random coil completely free from DNA in 2 M NaCl-8 M urea. The result shows that the formation of core particles leads to remarkable reduction in the degree of freedom of the histone molecular movement. In particular, the following phenomena were clearly observed: broadening of line width and decrease in the area of the peak region of δCH_2 (3.25 ppm) of arginine, αCH_2 (4.0 ppm) of glycine, and methyl groups (0.9 ppm or less) of nonpolar amino acid residues (leucine, isoleucine, and valine). The above-mentioned observation shows that these residues relate to the bond with DNA. In the condition of 0.6 M or less NaCl (a to c), a histone octamer bonds with DNA to form a core particle. Based on the NMR spectrum (f) of the histone in the state of random coil, the spectrum of the core particle (a to c) was analyzed. The analysis revealed the following: in the case of no addition of salt (a), the N-terminal and C-terminal regions of H2A and H2B do not bond with the DNA in the core particle to form a random coil structure; and in the presence of 0.3 to 0.6 M NaCl (b, c), the N-terminal regions of H3 and H4 do not bond with the DNA in the core particle additionally to form the random coil structure. On the other hand, with increase of NaCl concentration from 0.6 M to 2.0 M, the histone is dissociated from the DNA, with the result that the peaks (ring-current shifted peaks) appear in the region of 0 to 1 ppm

(c to e), the peaks being related to aromatic amino acid residues contained in the nonpolar site in the histone molecular center. Simultaneously, the acute resonance spectrum of the aromatic amino acid residues is observed in the low magnetic field region (6 to 10 ppm, not shown in FIG. V-10) with dissociation of histone. The difference between the spectrum (f) and spectrum (e) is owing, in the latter, to the effect of the formation of a complex that is conducted by each of histone molecules that have been dissociated from the DNA in 2 M NaCl. The above-mentioned facts clarify that in the interaction between the DNA and histone in the nucleosome core particle, the bond between the nonpolar site in the histone molecular chain and the DNA plays a major role.

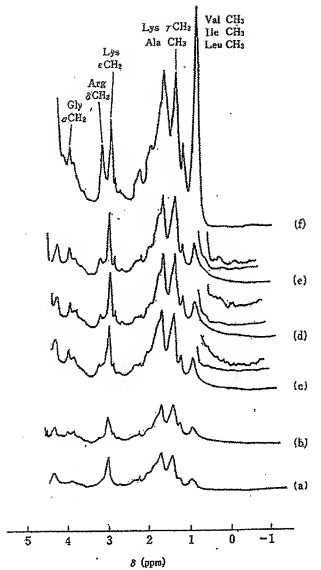

FIG. V-10 Salt concentration dependence of 270 MHz ^{1}H-NMR spectrum (high magnetic field region) in nucleosome core particle (Cary et al., 1978)

Solvent: 1 mM phosphate-D_2O buffer solution (pH 6.5)-NaCl, NaCl concentration (M): (a) 0, (b) 0.3, (c) 0.6, (d) 1.2, (e) 2.0. Spectrum (f) was measured in 2.0 M NaCl-8 M urea. (c), (d), and (e) include

spectra obtained by convolution difference in the region of 0 to 1 ppm.

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はその解析を進め，H2B の 40 位チロシンが H2A の 26 位プロリンと架橋していることを示した．

以上，ヒストン-ヒストン相互作用についていくつかの実験例をあげて述べてきたが，ほかにもアガロースゲル中での拡散沈降法（Johns & Forrester, 1975），免疫学的手法（Feldman & Stollar, 1977），アフィニティクロマトグラフィー（Mizon *et al.*, 1974）など各種の研究が行われているが，紙面の部分で省略する．（林　宏昭）

（ii） DNA-タンパク質間相互作用

ヌクレオソーム中の DNA-ヒストン間相互作用の解析には物理化学的手法と化学的手法があり，前者には核磁気共鳴法（NMR），熱融解法などが，後者には化学修飾法，架橋法，DNA 分解酵素を用いる方法などがある．また，これらの手法はヌクレオソームと非ヒストンタンパク質 HMG との相互作用の解析にも用いられている．ヌクレオソームの構造および DNA-ヒストン間相互作用については他書（大場，1978；McGhee & Felsenfeld, 1980 a；Kornberg & Klug, 1981；市村ら，1983；座間ら，1984）も参照されたい．

a） DNA-ヒストン間の結合

ヌクレオソームはクロマチンの基本単位構造であり，約 200 塩基対の DNA がヒストン H2A，H2B，H3，H4 各2分子よりなるヒストン八量体の外側を左巻きに2巻きし，巻き始めと巻き終りの部位に H1 ヒストンが1分子結合した構造体である．ヒストン八量体に 146 塩基対の DNA が 1.75 回巻きついた部分はヌクレオソームコアと呼ばれる．コア粒子間をつなぐ DNA はリンカーと呼ばれる．

146 塩基対のヌクレオソームコア DNA は，292 個のリン酸基の負電荷をもつ．一方，たとえば仔ウシ胸腺の場合，ヒストン八量体は塩基性アミノ酸残基の 220 個（116 個のリジン＋104 個のアルギニン）の正電荷と酸性アミノ酸残基の 74 個の負電荷，すなわち正味 220−74＝146 個の正電荷をもつ．つまり，ヒストン八量体にはコア DNA の負電荷のちょうど半数を中和するだけの正電荷が存在する．したがって，ヒストンと DNA の結合には静電的相互作用がまず考えられる．これを支持する事実として，1) 0.6M NaCl で H1，0.6M 以上の NaCl で H2A と H2B，1M 以上の NaCl で H3 と H4 が DNA から解離する（Ohlenbush *et al.*, 1967；Burton *et al.*, 1978；Ruiz-Carrillo & Jorcano, 1979），2) pH 2 以下で DNA とヒストンは解離する，3) 尿素あるいはエタノールなど

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の有機溶媒に対しては，DNA-ヒストン間の結合は安定である (Olins *et al.*, 1977 ; Zama *et al.*, 1978 b)，などがあげられる．しかし他の相互作用も無視できない．たとえば，尿素存在下では DNA からのヒストンの解離は著しく低い塩濃度 (~0.2 M NaCl) で起こる (Woodcock & Frado, 1977)．これは，尿素によってヒストン八量体の構造がゆるむと，DNA とヒストン間の結合が弱くなることを示している．疎水性相互作用や，特殊な立体配置に依存した水素結合の寄与が考えられる．

ヒストン分子には，各種アミノ酸の分子内分布の偏りがある．N-末端および C-末端，特に分子の N-末端側 1/3 が塩基性アミノ酸含有率が高く，分子中央部から C-末端側にかけての部分は疎水性アミノ酸や酸性アミノ酸も多く，通常の球状タンパク質と似たアミノ酸組成をもつ．1 次構造を反映してヒストン分子は両末端でランダム構造をとり，分子中央部から C-末端にかけて高次構造を形成する．各ヒストンの高次構造部分が相互作用してヒストン八量体を形成する．したがって，ヒストン八量体は α-ヘリックス含量の高い球状構造部分と，塩基性アミノ酸含量が高くランダム構造をとる高塩基性末端鎖 (長い N-末端鎖と短い C-末端鎖) 部分に大別できる．直感的には末端鎖が DNA-ヒストン間の主要結合部位と考えられるが，以下の実験は，ヒストン八量体の球状構造部分と DNA の相互作用のほうがむしろ強固であることを示している．1) 単離したヌクレオソームコアをトリプシン処理してヒストン末端鎖を選択的にとり除いても，ヒストンは安定に DNA と結合し，"粒子" 構造は保たれている (Grigoryev & Krasheninnikov, 1982)．2) トリプシン処理して末端鎖を除いたヒストンも未処理のヒストンも，天然のクロマチンと同じ塩濃度で DNA から溶離してくる (Palter & Albert, 1979)．3) NMR 法により，ヒストン末端鎖と DNA の結合はゆるいことが示された (Cary *et al.*, 1978, 実験例 8 参照)．また，塩基性アミノ酸の化学修飾法により，ヒストン八量体球状構造部分のアルギニン残基と DNA が強く結合していることが示された (Ichimura *et al.*, 1982, 実験例 9 参照)．

コアヒストンの N-末端側の塩基性領域のリジン残基は細胞の生理的条件に応じてアセチル化される．アセチル化反応により，リジン残基側鎖の ε-アミノ基の正電荷が消失し，同時にいくぶん大きなグループが導入されることになる．一方，酪酸はヒストンデアセチラーゼの阻害剤なので，5~10 mM の酪酸を含む培地で培養した細胞からヌクレオソームコアを単離すると，コア粒子中のヒストン N-末端近傍のリジン残基側鎖が高い割合でアセチル化された (hyperacetylation)，高アセチル化コア粒子が得られる．高アセチル化

コア粒子の構造やその安定性を調べることにより，DNA-ヒストン間相互作用に関する情報が得られる．

核磁気共鳴法

磁気モーメントをもつ核の集団を静磁場の中におくと，核 Zeeman 効果によって，磁気モーメントの大きさと磁場の強さに従った不連続なエネルギー準位に分布する．この準位の間隔に相当する電磁波を照射すると共鳴吸収が観測される現象を核磁気共鳴（NMR）という．共鳴吸収の測定には，照射する電磁波の周波数または静磁場の大きさの一方を固定し，一方を連続的に変えて共鳴点を記録する掃引法が一般的であったが，最近では一定の周波数の強いパルス磁場を与えて広い範囲のシグナルに対して同時に共鳴条件を満足させ，その時間応答をミニコンピュータを用いてフーリエ変換して，通常の周波数スペクトルを求める，パルスフーリエ変換法が主となっている．NMR スペクトルの4つのパラメータ，NMR 吸収線の位置（化学シフト），面積（強度），幅，分裂，によって調べる分子についての情報が得られる．位置は核の存在状態を反映する．面積は同じ化学的環境にある核の数に比例する．幅は核の運動の自由度が減ると広がる．分裂は近くの核磁気モーメントとの相互作用で生じる．タンパク質および核酸の NMR 一般，実験法に関しては文献（荒田，1976；曽良，京極，1977；Campbell & Dobson, 1979）を参照されたい．

一般に，タンパク質のランダム構造の部分は動きやすい（mobility が高い）ので，その部分の共鳴核は，鋭い共鳴シグナルを与える．しかし，その部分がほかの分子と接触して構造形成に直接関与すると，動きにくくなる（mobility が低くなる）ので，共鳴シグナルは幅広くなる．上に述べたように，ヒストン分子のアミノ酸配列には偏りがある．分子両末端の塩基性部分には，塩基性アミノ酸とともにグリシン，セリン，プロリンなど α-ヘリックスをつくりにくい残基が多い．これに対し，分子中央部の非極性部分は，非極性，芳香族，酸性のアミノ酸残基が多く，α-ヘリックスをつくる傾向がある．したがって，NMR を観測することによって，ヒストンのポリペプチド鎖のどの部分が，ヒストン-ヒストンおよびヒストン-DNA の相互作用に関与しているかを明らかにできる．

〔実験例 8〕 NMR 法による DNA-ヒストン間相互作用の解析（Cary *et al.*, 1978）

ヒストンの塩基性部分と非極性部分のアミノ酸のプロトンは別々の位置に共鳴線を生じる．この性質を利用して，塩濃度変化によるヒストン分子の DNA からの解離過程を NMR で追跡することにより DNA-ヒストン間の結合について解析した．仔ウシ胸腺か

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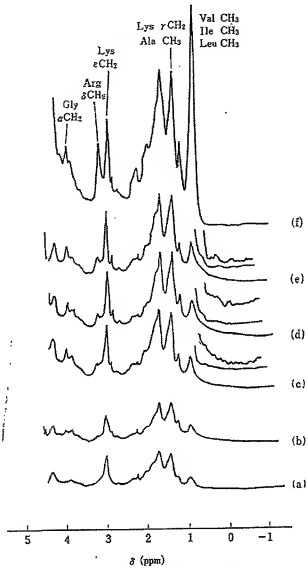

図 V-10 ヌクレオソームコア粒子の 270 MHz ^{1}H-NMR スペクトル（高磁場領域）の塩濃度依存性（Cary *et al.*, 1978）

溶媒：1mM リン酸-D_2O 緩衝液（pH 6.5)–NaCl, NaCl 濃度（M）：(a) 0, (b) 0.3, (c) 0.6, (d) 1.2, (e) 2.0. スペクトル（f）は 2.0M NaCl–8M 尿素中で測定した. (c), (d), (e) には, 0～1ppm の領域にスペクトル演算（convolution difference）により得たスペクトルが含まれている.

ら調製したヌクレオソームコア (3～5mg/ml) を 10mM リン酸緩衝液 (pH 6.5～7.0) に対し透析する．NMR 測定試料溶液を 0.5ml の D_2O–1mM リン酸緩衝液 (pH 6.5)–NaCl に対し 48 時間透析する．同じ透析を4回繰り返し，最後に 40 倍容量の同一の D_2O 緩衝液に対し透析する．NMR 測定は 270MHz フーリエ変換型の装置で行った．図V-10 に種々の塩濃度 (0～2M NaCl) 下で測定したヌクレオソームコアの ^{1}H-NMR スペクトルを示す．横軸は化学シフトで，問題となるプロトンピークと基準物質 2,2-ジメチル-2-シラペンタン-5-スルホン酸ナトリウム (DSS) のピークの共鳴周波数の差を分光計の共鳴周波数で割り，さらにこれを 10^6 倍して ppm 単位によって表す．コア粒子中のヒストンの NMR スペクトル (a) のピーク領域の全面積は，2M NaCl–8M 尿素中で DNA から完全に遊離したランダムコイル状態になったヒストンの NMR スペクトル (f) のそれと比較して 17～20% に減少する．これにより，コア粒子形成に伴いヒストン分子の運動の自由度が著しく減ることがわかる．特に，アルギニンの δCH_2 (3.25 ppm)，グリシンの αCH_2 (4.0ppm) および非極性アミノ酸残基 (ロイシン，イソロイシン，バリン) のメチルグループ (～0.9ppm) 線幅の広がりとピーク領域の面積の減少がはっきり観測され，これらの残基が DNA との結合に関与していることを示す．0.6M NaCl 以下 (a～c) ではヒストン八量体は DNA と結合してコア粒子を形成している．ランダムコイル状態になったヒストンの NMR スペクトル (f) を基準にして，コア粒子のスペクトル (a～c) を解析した．添加塩のないとき (a) は H2A と H2B の N-および C-末端鎖領域が，また，0.3～0.6M NaCl 存在下 (b, c) ではさらに H3, H4 の N-末端鎖領域が，コア粒子中で DNA と結合せずランダムコイル構造をとっていることがわかった．一方，0.6M から 2.0M に NaCl 濃度を増すと，ヒストンの解離に伴って，ヒストン分子中央部の非極性部位に含まれる芳香族アミノ酸残基に関連するピーク (ring-current shifted peaks) が，0～1ppm 領域に現れてくる (c～e)．同じく芳香族アミノ酸残基の鋭い共鳴スペクトルがヒストンの解離に伴って低磁場領域 (6～10ppm, 図V-10 には示してない) で観察される．スペクトル (f) と (e) の違いは，後者では 2M NaCl で DNA から解離したヒストン分子同士が複合体を形成している効果による．以上から，ヌクレオソームコア粒子中の DNA–ヒストン間相互作用においては，ヒストン分子鎖上の非極性部位と DNA との結合が主要な役割を演じていることがわかる．

ヒストン H1, H5 を除去したニワトリ赤血球クロマチンの ^{1}H-NMR 測定からも，

瓜谷郁三・駒野 徹
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